

Response of *Desulfovibrio vulgaris* to Alkaline Stress

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Abstract

The response of exponentially growing *Desulfovibrio vulgaris* Hildenborough (DvH) to pH 10 stress was studied using oligonucleotide microarrays and a study set of mutants deleted in genes suggested by microarray data to be involved in alkaline stress response. These data showed that the response of *D. vulgaris* to increased pH is generally similar to *E. coli*, but apparently controlled by unique regulatory circuits since the alternative sigma factors (sigma S and E) contributing to this stress response in *E. coli* appear to be absent in *D. vulgaris*. Genes previously reported to be up-regulated in *E. coli* were up-regulated in *D. vulgaris*, including three ATPase genes and a tryptophan synthase gene. Transcription of chaperone and protease genes (ATP-dependent Clp and La proteases, and DnaK) were also elevated in *D. vulgaris*. As in *E. coli*, genes involved in flagella synthesis were down-regulated. The transcriptional data also identified regulators, distinct from sigma S and E, likely part of a DvH-specific stress response system. Characterization of a study set of mutants deleted in genes implicated in alkaline stress response confirmed a protective involvement of sodium/proton antiporter NhaC-2, tryptophanase A, and two putative regulators/histidine kinases DVU0331 and DVU2580.

Introduction

Sulfate reducing bacteria (SRB) are ubiquitous in nature and play an important role in global carbon and sulfur cycling. Their habitat range includes freshwater, marine and hyper saline aquatic systems, cold oceanic sediments, the deep subsurface, hydrothermal vents, and hot springs (11, 26, 35). Although long thought to be relatively restricted in catabolic range, this functionally defined assemblage is now recognized to be remarkably versatile. SRB mediate the degradation of aromatic compounds once thought to be refractory to anaerobic degradation, including benzene (1,3,8,22,23) and reduce a variety of metals including radionuclides (19,23,35). For these reasons they have also been studied for possible utility in the bioremediation of environments contaminated with organic and metal pollutants.

Desulfovibrio vulgaris Hildenborough is one of the better characterized of SRB. This Gram-negative Deltaproteobacterium, isolated 60 years ago from clay soil in Hildenborough, Kent (United Kingdom) has served as one of the principal models for resolving the physiological and genetic basis of sulfate respiration. The recent completion of its genome sequence (13) has enabled genome-wide expression studies (5,27,13,36,37) that are now beginning to resolve its adaptive response to changing environmental parameters. Although this information is essential for predicting its behavior in possible applications to bioremediation, information about the range of conditions that support *D. vulgaris* growth or survival remains scarce.

Alkaline environments are common in nature (e.g., alkaline ground waters, lakes, intestinal segments of some higher organisms) and in sites contaminated by human activity (29). There are some data documenting the presence of SRB in alkaline environments (2), but there is little known about specific adaptive mechanisms of these bacteria or even whether mechanisms

common to better characterized organisms such as *Escherichia coli* and *Bacillus subtilis* are used by SRB. Adaptive strategies used by other microbes include: i) increased proton pumping by ATP synthase; ii) increased metabolic acid production through amino acid deaminases and sugar fermentation; iii) changes in cell surface properties; and iv) increased expression and activity of monovalent cation/proton antiporters (9,29,33). Among these strategies, monovalent cation/proton antiporters are thought to play a central role in alkaline pH homeostasis in many bacteria (29).

In order to better resolve similarities and differences in the adaptation of *Desulfovibrio* species, we used genome-wide transcription profiling to characterize the response of *Desulfovibrio vulgaris* to an upshift in the pH of its growth medium. Adaptive mechanisms suggested by transcriptional analysis were then examined by characterizing a study set of mutants deleted in genes implicated in the alkaline stress response. Together these analyses revealed a response system mechanistically similar to better-characterized species.

Material and Methods

Cell growth and pH upshift conditions. *D. vulgaris* was grown in LS4D medium supplemented with lactate and sulfate (13,27). Medium pH was adjusted to 10 by addition of KOH when the culture reached late exponential growth phase (ca. 0.4 OD₆₀₀). After 30, 60, 120 and 240 min of incubation, during which time the pH remained between 9.8 and 10.1, the cells were harvested for RNA isolation. To test the growth rate of mutants at different pH values, cells were grown in B3 medium (containing per liter, 0.1g NaCl, 0.1g of MgCl₂•6H₂O, 0.1g CaCl₂•2H₂O, 0.5g NH₄Cl, 0.1g KCl, 1.4g of Na₂SO₄, 1g of Na₂S, 0.001g of resazurine, 1ml of 1M

K₂HPO₄, 1ml of trace minerals, 1ml of Thauer's vitamins, 1ml of 1M cysteine and 1ml of 1M Na₂S. The following buffering agents were used: 25 mM sodium bicarbonate for pH 7; 50 mM Tris for pH 7.5; 8.0, and 50 mM glycine for pH 8.0 and 9.0. In all media 50 mM lactate and 40 mM sodium sulfate were used.

Analytical methods. The concentrations of organic acids (lactate, pyruvate, acetate, formate and fumarate) and inorganic ions (sulfate, phosphate) in culture media were determined using a Dionex 500 system equipped with an AS11HC column. In some cases the concentrations of organic acids were also measured on an HPLC equipped with a HPX 78 (Bio-Rad) column. Hydrogen concentrations were determined with a RGD2 Reduction Gas Detector (Trace Analytical) with 60/80 MOLE SIEVE 5A column (6' X 1/8'') with N₂ as carrier gas.

Mutant construction. Bacterial mutants and strains are listed in Table 1. The deletion mutant JW381($\Delta nhaC-2$) was constructed through marker exchange with a mutagenic plasmid as described elsewhere (4). Primers used for the mutagenic plasmid construction were: 5'-TATGGCAGATGTCAATGC CGAAGT-3', 5'-AAGACTGTAGCCGTACCTCGAATCTAATGTAGGCTCCAGTGGCCGA-3' (for the upstream region) and 5'-ACGGCTTCCACGTCAACTATCTCA-3', 5'-AATCCGCTCACTAAGTTCATAGACCGTAGGGAAGGGCTACCTGAG GC-3 (for the DNA region downstream of the gene). The kanamycin resistance marker replacing *nhaC-2* is also flanked by sequence bar codes unique to this deletion, with 5'-GCCGACAGAGCTTGAGATA-3' at the promoter proximal end of Kan marker and 5'-AGCCTGGAACAGCTATACAC-3' at the distal end.

Insertion mutant JW391 was constructed using plasmid pMO391 bearing spectinomycin resistance gene (Sp^R). pMO391 is pCR[®]8/GW/TOPO[®] (Invitrogen) with an internal *tnaA*

(DVU2204) fragment from *D. vulgaris* that was used to create an insertional mutation of *tnaA* through homologous recombination. Primers used for the strain construction were: *tnaA350Fd* (5'-ACA AGC CCG TCT TCA TCT CCA ACT-3') (forward) and *tnaA1273Rv* (5'- TGT AGT CCA TGT GGT CGT TGG TGT- 3'(reverse). The transposon mutants were generated by conjugation between *D. vulgaris* and *E. coli* BW20767 (pRL27) (19). The conjugation procedure was a modification of the method of Fu and Voordouw (12). Cultures of *D. vulgaris* were grown to mid exponential phase, and combined in a three or six to 1 ratio with the *E. coli* donor grown to early exponential phase in LC medium (1.0%[wt/vol] tryptone, 0.5% [wt/vol] yeast extract, and 0.5% [wt/vol] NaCl). Mating mixtures were concentrated by centrifugation. The concentrated cells were placed onto filter discs (0.22 µm pore diameter, GSWP, Millipore Billerica, MA) and the discs were placed on the surface of solidified LS4 (LS4D with 1% [wt/vol] yeast extract added) and incubated for sixteen hours at 34°C. Then the cells were washed from the membrane with 2ml LS4 medium. After six hours of incubation, antibiotic G418 (400 µg/ml) was added to select for the transposon mutants and nalidixic acid (200 µg/ml) was added to select against the *E. coli* donor. Then cells were spread onto LS4 agar (100 - 500 µl/plate) with both antibiotics. The plates were incubated in the anaerobic chamber at 34°C for at least four days to allow the colonies to grow. The chromosomal localization of the transposon insertions was identified by sequencing DNA after semi-random PCR amplification. For semi-random PCR, a variation of a protocol described by Chun et al. (7) was used. One microliter of a 50-µl boiled single-colony suspension in distilled H₂O was used as the template DNA in a 20-µl PCR mixture containing primer *tpnRL17-1* (5'-AAC AAG CCA GGG ATG TAA CG-3') (19) and either primer *CEKG 2A* (5'-GGCCACGCGTCGACTAGTACN10AGAG-3'), *CEKG 2B* (5'-GGCCACGCGTCGACTAGTACN10ACGCC-3'), or *CEKG 2C* (5'-

GGCCACGCGTCGACTAGTACN10GATAT-3'). One microliter of a 1:5 dilution of this reaction mixture was used as the template DNA for a second PCR performed with primers tpnRL17-2 (5'-AGC CCT TAG AGC CTC TCA AAG CAA-3') and CEKG 4 (5'-GGCCACGCGTCGACTAGTAC-3'). For the first reaction, the thermocycler conditions were 94°C for 2 min, followed by six cycles of 94°C for 30 s, 42°C for 30 s (with the temperature reduced 1°C per cycle), and 72°C for 3 min and then 25 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 3 min; for the second reaction, the thermocycler conditions were 30 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 3 min. Samples that produced distinct bands on an agarose gel after the second reaction were cleaned with a PCR purification kit (Qiagen, Valencia, CA) and sequenced by using primer tpnRL17-1. The chromosomal locations of the insertions were identified by BLAST analysis of the sequences adjacent to the transposon compared with the complete genome.

Microarray construction, hybridization, and image analysis. DNA microarrays covering 3,482 of the 3,531 annotated protein-coding sequences of the *D. vulgaris* genome were designed, constructed, and validated with 70mer oligonucleotide probes as previously described (5,13,27). Briefly, all oligonucleotides were commercially synthesized without modification by MWG Biotech Inc. (High Point, NC), prepared in 50% vol/vol DMSO (Sigma-Aldrich, St. Louis, MO), and spotted onto UltraGAPS glass slides (Corning Life Sciences, Corning, NY) using a BioRobotics Microgrid II microarrayer (Genomic Solutions, Ann Arbor, MI). Each oligonucleotide probe had two replicates on a single slide. After printing, the oligonucleotide probes were fixed onto the slides by UV cross-linking (600 mJ of energy) according to the protocol of the manufacturer of the UltraGAPS glass slides (Corning Life Science).

Total RNA extraction, purification, and labeling were performed independently on each cell sample using previously described protocols (5, 13). Briefly, total cellular RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA extracts were purified according to the RNeasy Mini Kit (Qiagen Valencia, CA) instructions and on-column DNase digestion was performed with the RNase-free DNase Set (Qiagen) to remove genomic DNA contamination according to the manufacturer's procedure. Labeling of cDNA targets from purified total RNA was carried out using the reverse transcriptase reaction with random hexamer priming, and the fluorophore Cy5-dUTP (Amersham Biosciences, Piscataway, NJ). Genomic DNA was extracted from *D. vulgaris* cultures at stationary phase and labeled with the fluorophore Cy3-dUTP (Amersham Biosciences). The efficiency of labeling was routinely monitored by measuring the absorbance at 260 nm (for DNA concentration), 550 nm (for Cy3), or 650 nm (for Cy5).

To hybridize a single glass slide, the Cy5-dUTP-labeled cDNA probes obtained from stressed or unstressed cultures were mixed in equal amounts with the Cy3-dUTP-labeled genomic DNA (5, 27). After washing and drying, the microarray slides were scanned using the ScanArray Express microarray analysis system (Perkin Elmer). The fluorescent intensity of both the Cy5 and Cy3 fluorophores was analyzed with the software ImaGene version 6.0 (Biodiscovery, Marina Del Rey, CA).

Microarray analysis. Arrays were scanned using the scanning laser confocal fluorescence microscope of the ScanArray® Microarray Analysis System (GSI Lumonics), and hybridization signal intensities were quantitated using the software of ImaGene™ (Biodiscovery). Statistical analysis of the microarray data was performed using ArrayStat (for

details, see Ref. 5) and cluster analysis was performed using TIGR MultiExperiment Viewer (MeV). For raw microarray data see <http://vimss.lbl.gov/pH10Stress/microarray-rawdata>.

Protein sequence analysis. For protein sequence analysis programs Tmpred:

http://www.ch.embnet.org/software/TMPRED_form.html (15), LipoP 1.0 Server:

<http://www.cbs.dtu.dk/services/LipoP> (17), and SignalP 3.0 (10) were used from the Expasy data

base. Genome and open reading frames of *D. vulgaris* and gene content of different sequenced

genomes were analyzed using Microbesonline (<http://www.microbesonline.org/>) and NCBI

databases.

Results and Discussion

General features of the physiological and transcriptional response to elevated pH.

Initial studies examined the general physiological response of *D. vulgaris* to an abrupt increase in pH (Fig. 1). Lactate consumption stopped immediately following the pH shift, as did production of the metabolites, hydrogen and acetate (Fig. 1C). However, viable cell numbers remained constant during the two-hours following the shift (Fig. 1A), as demonstrated by enumerating cells on agar plates and immediate resumption of growth measured by optical density following transfer to fresh neutral medium. During this period of exposure to elevated pH, altered expression of approximately 400 genes was observed. At 30, 60, 120 and 240 min following the shift, transcription of 78, 122, 178, 184 genes, respectively, was significantly up-regulated (log base 2 of signal intensity change greater than 1 and Z-score greater than 2). The transcription of a comparable number of genes was down-regulated by at least 2 fold at the same time points (175, 267, 210 and 183 genes, respectively). The majority of the up-regulated genes fell into the following COG functional categories: amino acid biosynthesis, energy metabolism, and signal transduction systems. A large fraction of the down-regulated genes were assigned to signal transduction, transcription, and phage related categories.

Using K-means clustering analysis, all genes were assigned to 30 groups based on their patterns of expression. Graphs representing all groups are shown in Fig. S1. Genes comprising each group are listed in Table S2. Cluster 22 (44 genes) is comprised of those genes most highly up-regulated at all time points, whereas cluster 19 consists of those that were only moderately up-regulated (122 genes). The most highly down regulated genes are collected in cluster 4 (57 genes), and those moderately down regulated (100 genes) comprise cluster 21.

Genes up-regulated during exposure to high pH. Elevated pH is generally thought to stress the cell through alkalinization of the cytoplasm, reduction of membrane potential, and damage to protein and the cell envelope (9,33, reviewed in 29,31). The cell responds by pumping protons inside, importing or synthesizing compounds to acidify the cytoplasm, and activating systems of protein repair or degradation. These are all reflected in the response of *E. coli* to elevated pH. Highly up-regulated genes correspondingly up-regulated in *D. vulgaris* include: ATPase synthase, Na^+/H^+ antiporter NhaC-2 (DVU3108), genes encoding chaperones and proteases such as DnaK (DVU0811, $\log_2 R = 0.8 - 1.5$), ATP-dependent Clp protease subunit B (DVU1874, $\log_2 R = 1.2-1.5$), and ATP-dependent protease La (DVU3303). In *D. vulgaris* these genes demonstrated the highest increase in expression at all time points ($\log_2 R$ from 0.54 to 3.7 and average $\log_2 R \approx 2$) and a tendency to increased levels of expression at 240 min of stress (cluster 22) or were moderately up-regulated (average $\log_2 R \approx 1$) at all time points (cluster 19), as determined by the K-means cluster analysis (see Tables 2 and S2).

The *D. vulgaris* gene coding for a putative Na^+/H^+ antiporter NhaC-2 (DVU3108) is of particular interest. The role of Na^+/H^+ antiporters in regulation of intracellular pH homeostasis and survival at high pH has been demonstrated for a number of bacteria (6,16, 28, 29). Although several genes in *D. vulgaris* have been identified as putative Na^+/H^+ antiporters, only the expression of *nhaC-2* changed substantially in response to high pH. Our analysis of a *nhaC-2* deletion mutant demonstrated an increased sensitivity to pH 8.9, relative to the wild type, that was accentuated at higher concentrations of sodium chloride (Fig. 2 and 3). Increased cell lysis in stationary phase was observed for this mutant, but not for the wild type (Figure 3B,D). This phenotype is similar to that of an *E. coli nhaA* deficient mutant (29).

According to the *D. vulgaris* genome sequence, *nhaC-2* is transcribed in the same direction as DVU3110, an upstream gene (also highly up-regulated at high pH) encoding a putative FAD-binding oxidoreductase/L-aspartate oxidase with a *bona fide* homolog present only in one other *Desulfovibrio* strain (*D. vulgaris* DP4, >99% identity). The next most closely related amino acid sequence (42.8% identity) is found in the genome of *Magnetospirillum magneticum* AMB1, coding for a member of the succinate dehydrogenase/fumarate reductase protein family (COG1053). Also, the amino acid sequence of DVU3110 demonstrates 29.5% identity to the *E. coli* L-aspartate oxidase (b2574, NadB). The absence of genes typically associated with characterized succinate dehydrogenases/fumarate reductases, such as genes for b type cytochromes and small membrane anchor proteins in the vicinity of DVU3110 does not allow us to suggest that DVU3110 codes for a protein with similar activities. In addition to that, neither fumarate or succinate was detected in cultural medium of *D. vulgaris* exposed to pH 10 (data not shown). Nonetheless, the presence of a 4Fe-4S ferredoxin (DVU3109) immediately down stream of DVU3110 suggests that the latter might encode for an oxidoreductase. An identical gene context surrounds the DVU3108 homolog in *D. vulgaris* DP4 (NCBI NC008751.1). Since an ortholog of DVU3110 has not been identified in the sequenced genome of another *Desulfovibrio* species (*D. desulfuricans* G20), a response to elevated pH via the combined activities of the oxidoreductase/L-aspartate oxidase and NhaC may be a species-specific strategy. Of additional note, genes identified as L-aspartate oxidase in the genomes of many Gammaproteobacteria, for instance *E. coli*, *Nitrosococcus oceanii* and *Methylococcus capsulatus*, are immediately upstream of a gene for a putative sigma E, suggesting a more general involvement of such type of reductase/L-aspartate oxidase in stress response.

In addition to increased transcription of the gene encoding L-aspartate oxidase, a number of genes involved in amino acid synthesis and metabolism were also consistently up-regulated. Tryptophan synthase subunits A and B (DVU0471, DVU0470) and other members of the tryptophan operon (DVU0460-469) were slightly or moderately up-regulated (Table 4). In addition, transcription of a gene annotated as tryptophanase (DVU2204) was elevated after 240 min of pH stress ($\log_2 R = 1.3$). The orthologous gene in *E. coli* (*tnaA*) was previously shown to be up-regulated at pH 9 (25, 34) and growth of a *D. vulgaris* mutant deleted for this gene was diminished at pH 8 (data not shown). The concerted increase in transcription of genes for biosynthesis and transport of amino acids other than tryptophan (genes for cysteine synthase A, dihydrodipicolinate reductase involved in lysine biosynthesis, isopropylmalate dehydratase involved in leucine biosynthesis, homoserine dehydrogenase involved in aspartate biosynthesis) suggests that *D. vulgaris* employs multiple components of amino acid metabolism for survival at high pH (Table 4).

The integrity of the cell envelope is also challenged at elevated pH. Changes in the expression of several genes involved in cell wall and membrane biogenesis were differentially expressed following the increase in the pH of the growth medium. Increased transcription of a gene encoding the heptosyltransferase family protein (DVU1446), involved in the synthesis of the inner core region of lipopolysaccharide, was a notable example (Table 2). One of the most highly up-regulated genes associated with cell envelope structure was *fabZ* (DVU2368), encoding a putative beta-hydroxyacyl-(acyl-carrier-protein) dehydratase. This gene, involved in fatty acid biosynthesis, is part of a six-gene operon containing, in addition to *fabZ*, genes encoding UDP-N-acetylglucosamine O-acyltransferase (DVU2367), UDP-3-O-(R-3-hydroxymyristoyl)-glucosamine N-acyltransferase (DVU2369), outer membrane protein OmpH

(DVU2370) and N-acetylmuramoyl-L-alanine amidase (DVU2371). The first two enzymes are involved in lipopolysaccharide biosynthesis, while the latter two are part of the peptidoglycan biosynthetic pathway. Transcription of two genes in this operon (DVU2369 and DVU2370) was significantly increased at pH 10 ($\log_2 R$ of 0.8-1.0 and 1.6-1.8, respectively). The *fabZ* gene in *E. coli* is part of the sigma E regulon, exhibiting a sigma E consensus promoter, and is up-regulated following heat shock (30). Although the expression of this gene in *E. coli* was reported not to be altered by either an increase or decrease in pH, in *D. vulgaris* the transcription of all genes in the operon containing the *fabZ* ortholog increased following the shift to higher pH. Changes in the expression of these genes in *D. vulgaris* have not been detected in response to other environmental stressors, including salt, nitrate, potassium chloride and heat shock (5, 13, 27, Wall unpublished data).

Multiple genes associated with energy generation and electron transfer reactions showed increased expression at elevated pH. For instance, DVU0692, DVU0693, and DVU694 were significantly up-regulated after 30 min of pH stress. These were previously annotated as subunits of a molybdopterin oxidoreductase of unknown specificity. Analysis of amino acid sequences predicted that the proteins encoded by DVU0694 (the first gene in the operon) and DVU0692 contain two and ten transmembrane helices, respectively. Since they both also contain putative signal peptide sequences, it is likely that together they comprise a membrane bound protein complex. The transcription of several other genes predicted to be involved in energy generation and electron transfer reactions also increased. These included genes in cluster 2 encoding the large subunit of the periplasmic NiFe hydrogenase isozyme 2 (DVU2526) and the Fe-S subunit of glycolate/lactate oxidase (DVU3028). In addition, genes for formate dehydrogenases (DVU0587, DVU0588, DVU2481 and DVU2482), thiosulfate reductase

(DVU0179) and CO-induced hydrogenase (DVU2286-2291) were moderately up-regulated.

Together these trends suggested a redirection of electron flow from sulfate reduction to unknown electron acceptors, possibly to maintain cytoplasmic redox status during the stress.

A number of genes showing differential expression during alkaline pH stress code for proteins of unknown function (Table 2). BLAST analysis did not reveal any homologous proteins in publicly available databases. Thus, their possible role in surviving at high pH is obscure. Among the genes most highly up-regulated following 240 min at pH 10 were DVU3300 and DVU3301 ($\log_2 R = 3.70$ and 3.57 , correspondingly). These most likely comprise an operon containing two additional genes, DVU3298, DVU3299, that were also up-regulated at alkaline pH (Figure 4). Amino acid sequence analysis predicted that the proteins encoded by these genes possess at least one transmembrane segment and a signal peptide, suggesting a membrane association. These genes were also previously shown to be up-regulated in *D. vulgaris* in response to acid, nitrate, nitrite and sodium chloride stress (13, 27, Wall unpublished data).

Changes in expression of signal transduction and regulatory genes. The affect of high pH on genes categorized as signal transduction genes revealed that of the 273 genes in this category, 87 showed significant differential expression for at least one time interval, as judged by a Z-score greater than 1.5. The Z-score was greater than 2 for 44 of them. A peculiar feature of the *D. vulgaris* genome is the relatively large number of open reading frames annotated as methyl-accepting chemotaxis proteins (MCPs). For example, *E. coli* has 5 MCP genes versus 27 in *D. vulgaris*. In *E. coli* these proteins are involved in chemotactic responses, transducing signals to the flagellar motor. Three of *D. vulgaris* MCP genes were up-regulated at pH 10. One

(DVU3035) is located in close proximity to an operon comprised of genes presumably involved in assimilation of lactate and its conversion to acetate, a central metabolic pathway in *D. vulgaris*. This gene was previously shown to be up-regulated with salt stress (27). A second (DVU3082) was up-regulated two fold at 120 min and 240 min of alkaline pH exposure. This gene was previously shown to be up-regulated with acid stress but unaffected by other stressors so far examined for *D. vulgaris*. The third (DVU1884), up-regulated two fold after 240 min of alkaline pH, was previously shown to be also up-regulated with heat shock and nitrate stress (5, 13). Interestingly, this latter gene is immediately downstream of a locus of 14 genes (including ClpB, Dna J and peptidyl-prolyl *cis-trans* isomerase) associated with the *E. coli* general stress response. Although, expression of flagellar genes in *D. vulgaris* was decreased and motility of the cells was suppressed, the MCPs may be involved in regulation of cellular responses other than motility. For instance, it was recently demonstrated that a chemosensory-like pathway regulates developmental gene expression in *Myxococcus xanthus* (18).

The gene DVU0667 coding for a HD domain protein was among the most highly up-regulated genes in cells exposed to pH 10 for 30 min. Proteins with the HD motif belong to a superfamily of metal-dependent phosphohydrolases that include a variety of uncharacterized proteins associated with nucleotidyltransferases and helicases from *Bacteria*, *Archaea*, and eukaryotes. Increases in expression of these genes are suggestive of adaptive changes associated with unidentified regulatory pathways that affect RNA modifications and stability in response to the pH upshift.

There are only a few genes encoding alternative sigma factors in *D. vulgaris* (sigma N, sigma H and a flagellar sigma factor). As reported by Chhabra et al. (5), neither a gene for sigma E or RpoS have been found in the genome of this bacterium. This suggests that *D. vulgaris* may

respond to stresses that cause misfolding or degradation of cell envelope proteins differently from better studied bacteria. Indeed, in addition to the absence of sigma E, there are no *deg* and *rse* orthologs recognized in the genome. These have been shown to play an important role in the response of *E. coli* to periplasmic stress (30, 31). Thus *D. vulgaris* apparently uses a system distinct from that of *E. coli* to control expression of genes encoding proteases and chaperone-like proteins. One of the most highly up-regulated genes (DVU3303) encodes a protease homologous to Lon, but having a very peculiar domain structure. It contains a signal receiver domain common to CheY, OmpR, NtrC, and PhoB, and a phosphor-acceptor site for histidine kinase homologs. Its carboxyl terminus contains ATPase and a Lon protease (S16) proteolytic domains (Fig. 4). Such domain structure suggests that this protease can be activated by phosphorylation and transfers the signal to an unknown protein which might be subsequently functionally altered by cleavage. Immediately downstream of DVU3303 are genes for a histidine kinase (DVU3304) and a putative response regulator (DVU3305), making this gene cluster a plausible candidate for a regulatory circuit involved in pH stress response and other stresses, since this regulatory genes were shown to be also up-regulated under pH 5.5, nitrate, chromium, or sodium chloride stress (13, 27, Wall et al. unpublished data).

Two additional genes annotated as histidine kinases (DVU0331 and DVU2580) were also up-regulated in response to elevated pH. Mutants constructed for these two genes, JW3011 and JW3024, demonstrated increased sensitivity to elevated pH (Fig. 2). Since the genes encoding these kinases are proximal to genes for cell wall biogenesis, they may also be part of a more general *D. vulgaris* response to cell envelope stress (Fig. 5 and 6).

Genes transiently up-regulated during exposure to high pH. Thirty two genes demonstrated a more complex pattern of expression change following adjustment to pH 10 – greatest expression was observed at 30 min , followed by a relative decrease at 60 min, and increasing again at 120 min (cluster 18 Table S2). Since, most of these are located in chromosomal segments containing phage-related genes, this pattern of expression may reflect a response system specific to phage biology.

Genes down-regulated during exposure to high pH. There were 57 genes significantly down regulated (cluster 4, $\log_2 R$ from -1.5 to - 3.6 and average $\log_2 R \approx - 2$) and 100 genes moderately down regulated (cluster 21, average $\log_2 R \approx - 1$) during the pH 10 stress (Table 3 and S2). The most highly down-regulated gene at 240 min was DVU2725, coding for a membrane protein of unknown function. It is located within the cluster of the phage-related genes and demonstrates high homology (48%) to a gene from *Chromobacterium violaceum* ATCC 12472 which is also located in a phage gene cluster. The down regulation of 28 genes annotated as phage or transposon related suggests that control of their expression is an important aspect of the *D. vulgaris* stress response (Table 3).

Genes for peptidyl-prolyl *cis-trans* isomerase (DVU2569, $\log_2 R = -1.4 - - 1.8$), FKBP-type protein-L-isoaspartate O-methyltransferase (DVU1849, $\log_2 R = -1.2 - - 1.4$); and peptidyl-prolyl *cis-trans* isomerase B-2 (DVU1873, $\log_2 R = -0.8 - - 1.3$) were found to be moderately down regulated with alkaline stress. All three proteins are involved in cell wall biosynthesis, and observed changes in their expression may reflect a pause in cell growth and (or) adaptive changes in cell wall composition to the stress. In addition, some energy production and central metabolism genes were consistently down-regulated, including pyruvate carboxylase (DVU1834,

$\log_2 R = -1.4 - -1.8$), B₁₂ binding domain protein/radical SAM domain protein (DVU3016, $\log_2 R = -1.5 - -2.1$), desulfoferredoxin (DVU3183, $\log_2 R = -1.0 - -1.2$); ferredoxin II (DVU0305, $\log_2 R = -1.5 - -1.9$), and L-lactate permease family protein (DVU2451, $\log_2 R = -1.1 - -1.8$). The *hupD* gene (DVU1923, $\log_2 R = -0.6 - -1.6$) for hydrogenase expression/formation was down regulated after 240 min of pH 10 stress.

Relationship to other microorganisms and alternative stress response systems. The model shown in Figure 7 draws upon the data presented in this study to provide an overview of the response *D. vulgaris* to alkaline stress. The observed increase in expression of genes for ATPase synthase and the Na⁺/H⁺ antiporter NhaC-2 serves to attenuate alkalinization of the cytoplasm via increased proton import, and is similar to the response of *E. coli* and other bacteria (20, 25, 29). Similarly, a decrease in expression of flagellar genes and presumptive acidification of the cytoplasm via increased expression of genes involved in amino acid metabolism are also shared responses. Increased expression of several genes for proteins potentially involved in energy generation or electron transfer reactions was also observed. These included genes for a formate dehydrogenase, a molybdopterin oxidoreductase, cytoplasmic *coo*-hydrogenase and a periplasmic NiFe hydrogenase. These latter changes may be part of a more general strategy to retain a cellular redox state necessary to sustain cellular functions when lactate consumption and sulfate respiration are repressed.

Although growth was arrested at pH 10, the cells remained metabolically active, as assessed by rescue in culture and a complex transcriptional response during the period of exposure. As part of this response there appeared to be a modification of the cell envelope, indicated by a modulation in the expression of genes involved in cell wall biosynthesis. Some

genes in this category showed decreased expression, whereas others were up-regulated. We anticipate that ongoing studies of the cellular proteome and membrane composition will better elucidate the character and possible function of any changes in membrane structure.

Finally, our observations suggest that, although the response of *D. vulgaris* to increased pH is similar to *E. coli*, this response is controlled by unique regulatory circuits. The alternative sigma factors (sigma S and E) contributing to this stress response in *E. coli* are apparently absent in *D. vulgaris*. Our transcriptional analysis has identified several regulators that are likely part of a *D. vulgaris*-specific stress response system. These now provide specific targets for continued biochemical and genetic characterization of the stress response system of this organism and its relationship to regulatory pathways in other bacteria, including the diversity of *Desulfovibrio* now represented in the family *Desulfovibrionaceae*.

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Figure Legends

Figure 1. Growth and pH changes during pH up-shift. Panel A – cell numbers; Panel B – pH; Panel C – lactate and acetate concentration in the growth medium of the stressed culture.

Figure 2. Growth characteristics of the selected *D. vulgaris* mutants at elevated pH with 50mM glycine buffer. A - pH 7.0; B - pH 7.9; C - pH 8.9.

Figure 3. Growth characteristics of the selected *D. vulgaris* mutants at elevated pH and sodium chloride concentration with 50 mM Tris buffer. A – pH 7.0 and 40 mM NaCl; B – pH 7.9 and 40 mM NaCl; C – pH 7.0 and 80mM NaCl; D – pH 7.9 and 80 mM NaCl.

Figure 4. Most highly expressed operons during pH stress, encoding a putative Lon protease (DVU3303), two regulators (DVU3304 and DVU3305), and set of membrane proteins of unknown function (DVU3299-3301). Color of the genes depicts changes in expression: white – up-regulation, grey – unchanged expression. Abbreviations for domains for the Lon protease:

Lon - Lon protease domain, Rec - receiver domain, AAA - ATPase domain, Lon C - Lon protease C-terminal proteolytic domain.

Figure 5. Gene organization of chromosomal segment containing the gene for histidine kinase DVU0331 up regulated at high pH. Color of the genes depicts changes in expression: black – down-regulation, white – up-regulation, grey – unchanged expression.

Figure 6. Gene organization of a chromosomal segment containing the gene for signal transduction histidine kinase DVU2580 up regulated at high pH. Color of the genes depicts changes in expression: black – down-regulation, white – up-regulation, grey – unchanged expression.

Figure 7. Conceptual model of *D. vulgaris* response to high pH stress. Arrows near protein/enzyme/pathway names indicate change in corresponding gene expression.

Tables

Table 1. Strains of *D. vulgaris* Hildenborough used in this study.

Strain	Putative gene	Annotation	Strain description	Source
29579	Not applicable	wild type	Parent of deletion and plasmid insertion mutants	ATCC
JW381	DVU3108	<i>nhaC</i>	$\Delta nhaC::ntp$, MP ⁺	This study
JW391	DVU2204	<i>tnaA</i>	<i>tnaA::pMO391</i> ^b , MP ⁺	This study
JW3024	DVU2580	regulator	Tn5-RL27 insertion	This study
JW3011	DVU0331	histidine kinase	Tn5-RL27 insertion	This study
JW801	Deleted for megaplasmid (MP) ^a	wild type	Parent strain of transposon mutants	G. Voordow

^a MP: 202 kb megaplasmid endogenous to the *D. vulgaris* Hildenborough ATCC 29579

^b pMO391 is pCR[®]8/GW/TOPO[®] (Invitrogen) with an internal *tnaA* fragment from *D. vulgaris* used to create an insertional mutation through homologous recombination; Sp^R

^c Tn5-RL27 is a modified high frequency insertion transposon (19)

Table2. Highly upregulated genes during pH 10 stress (Cluster 22).

Gene ID	Gene description	Gene expression change, Log ₂ ratio			
		30min	60min	120min	240min
DVU0085	tryptophan synthase, beta subunit (trpB)	1.36	1.90	1.75	2.45
DVU0086	hypothetical protein	1.18	0.84	1.88	3.00
DVU0211	conserved hypothetical protein	1.32	1.68	1.02	1.94
DVU0258	sensory transduction histidine kinase-related	0.96	0.95	1.24	2.28
DVU0265	25.3 kd protein in hmc operon	1.61	2.08	1.54	1.56
DVU0303	hypothetical protein	1.58	1.87	2.05	1.82
DVU0331	putative histidine protein kinase	1.36	1.18	1.83	2.23
DVU0493	hypothetical protein	1.47	1.81	1.45	1.61
DVU0667	HD domain protein	2.28	1.52	1.74	1.69
DVU0693	respiratory nitrate reductase, beta subunit (narH)	1.09	1.52	1.48	2.01
DVU0694	molybdopterin oxidoreductase, molybdopterin binding subunit	2.17	1.91	1.01	1.97
DVU0774	ATP synthase, F1 epsilon subunit (atpC)	1.77	2.57	2.20	3.21
DVU0775	ATP synthase, F1 beta subunit (atpD)	1.11	1.55	1.63	2.27
DVU0776	ATP synthase, F1 gamma subunit (atpG)	0.87	1.60	1.13	2.26
DVU0855	coenzyme pqq synthesis protein, putative	1.64	1.76	1.60	1.96
DVU1035	glucokinase (glk)	1.09	2.03	2.11	1.82
DVU1198	riboflavin synthase, beta subunit (ribH)	1.94	2.05	1.59	2.03
DVU1304	ribosomal protein L4/L1 family	1.53	1.67	1.46	1.66

DVU1314	ribosomal protein L24 (rplX)	ND	1.20	1.41	2.41
DVU1319	ribosomal protein L18 (rplR)	1.66	1.64	1.34	1.80
DVU1322	ribosomal protein L15 (rplO)	1.51	1.64	1.80	2.34
DVU1370	hypothetical protein	1.73	1.83	1.60	1.45
DVU1446	Heptosyltransferase family	2.98	2.30	2.56	1.63
DVU1858	'Cold-shock' DNA-binding domain protein	1.56	1.68	1.58	2.31
DVU2283	hypothetical protein (3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase (fabZ)	1.24	1.56	1.64	1.64
DVU2368	outer membrane protein OmpH, putative	1.64	1.90	1.87	2.42
DVU2370	periplasmic (nife) hydrogenase large subunit	1.58	1.80	1.62	1.64
DVU2526	ferrous iron transport protein A, putative	1.68	1.24	1.42	1.98
DVU2572	efflux system protein	1.25	1.36	1.80	1.76
DVU2816	hypothetical protein	1.04	1.80	1.87	1.95
DVU2946	glycolate oxidase iron-sulfur subunit	1.98	2.45	2.10	2.16
DVU3028	methyl-accepting chemotaxis protein, putative	1.57	2.07	1.92	2.30
DVU3035	integral membrane protein, putative	1.41	1.74	1.31	1.56
DVU3081	Na ⁺ /H ⁺ antiporter NhaC (nhaC)	0.27	2.38	1.35	2.64
DVU3108	L-aspartate oxidase, putative	1.36	1.60	1.41	2.10
DVU3110	hypothetical protein	0.91	1.19	1.35	2.45
DVU3298	hypothetical protein	1.38	1.86	1.09	2.15
DVU3300	hypothetical protein	0.68	2.27	0.84	3.57
DVU3301	hypothetical protein	0.88	3.36	1.53	3.70
DVU3325	hypothetical protein	1.27	2.13	2.12	1.94
DVU3371	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	0.54	2.18	1.55	1.87

Log₂ gene expression ratio at minutes after pH was changed vs control at the same time

ND - expression was not detected

Table 3. Most down regulated genes during pH 10 stress (Cluster 4)

Gene ID	Gene description	Gene expression change, Log ₂ ratio				
		30 min	60 min	120 min	240 min	ptr*
DVU0475	membrane protein	-2.43	-2.23	-2.91	-2.49	Y
DVU0555	hypothetical protein	-3.09	-2.02	-2.90	-1.98	Y
DVU0559	lipoprotein, putative	-2.35	-2.36	-3.49	-1.98	Y
DVU0561	glycosyl transferase, group 1 family protein	-3.21	-2.83	-3.16	-2.30	Y
DVU0562	ISD1, transposase OrfA	-2.75	-1.95	-2.33	-1.53	Y
DVU0618	hypothetical protein	-2.01	-2.05	-2.40	-1.64	
DVU0817	hypothetical protein	-2.18	-1.99	-2.04	-1.84	
DVU0820	hypothetical protein	-2.36	-1.74	-2.18	-1.63	
DVU0821	conserved hypothetical protein	-3.76	-3.34	-3.20	-2.55	
DVU0822	hypothetical protein	-2.34	-2.32	-2.50	-0.93	
DVU1010	hypothetical protein	ND	-2.12	-2.24	ND	
DVU1014	hypothetical protein	-2.40	-2.13	-2.28	-2.36	
DVU1015	hypothetical protein	-1.53	-2.20	-2.67	-1.27	
DVU1166	hypothetical protein	ND	-2.00	-2.92	-1.68	
DVU1477	hypothetical protein	-1.76	-2.15	-2.20	-1.83	Y
DVU1478	hypothetical protein	-2.40	-2.08	-2.14	-2.04	Y
DVU1509	conserved hypothetical protein	-2.61	-2.57	-3.15	-2.86	Y

DVU1510	hypothetical protein	-2.46	-2.41	-2.76	-2.44	Y
DVU1519	transcriptional regulator	ND	-2.88	-3.29	-1.84	Y
DVU1520	hypothetical protein	ND	-1.74	-2.50	ND	Y
DVU1691	hypothetical protein	-2.30	-2.21	-2.39	-1.80	
DVU1706	hypothetical protein	-2.37	-2.02	-3.22	-2.58	Y
DVU1711	hypothetical protein	-2.40	-2.00	-2.41	-2.40	Y
DVU2007	nuclease, putative	-1.57	-1.97	-2.52	-1.77	
DVU2010	ISD1, transposase OrfB	-2.07	-2.09	-2.16	-1.90	Y
DVU2106	sigma-54 dependent transcriptional regulator	-1.85	-1.99	-2.65	-2.12	Y
DVU2174	hypothetical protein	-2.08	-2.07	-2.71	-1.68	Y
DVU2177	hypothetical protein	-1.53	-2.26	-2.67	-2.26	Y
DVU2178	ISDvu2, transposase OrfB	-1.48	-2.17	-2.09	-0.40	Y
DVU2179	ISDvu2, transposase OrfA	-2.37	-2.34	-2.66	-2.21	Y
DVU2200	hypothetical protein	-2.04	-2.24	-2.57	-2.56	
DVU2219	hypothetical protein	-2.25	-2.35	-2.80	-1.50	
DVU2220	conserved hypothetical protein	-2.11	-1.83	-2.04	-1.65	
DVU2265	hypothetical protein	-2.51	-1.77	-1.88	-2.12	
DVU2430	RNA-binding protein	-2.54	-2.00	-2.33	-1.37	
DVU2655	D-alanyl-D-alanine carboxypeptidase	-2.29	-2.64	-1.82	-1.88	
DVU2686	peptidase, S24 family	-2.66	-2.31	-2.69	-2.11	Y
DVU2696	conserved hypothetical protein	-3.05	-3.27	-2.96	-2.69	Y
DVU2697	hypothetical protein	-2.15	-2.43	-1.99	-1.97	Y
DVU2709	hypothetical protein	-1.85	-2.76	-2.89	ND	Y
DVU2725	membrane protein, putative	-3.27	-3.28	-3.65	-3.66	Y
DVU2836	hypothetical protein	-2.70	-2.18	-2.87	-2.19	Y
DVU2839	conserved hypothetical protein	-1.98	-1.72	-2.17	-2.47	Y
DVU2840	conserved hypothetical protein	-2.15	-1.64	-2.60	-2.08	Y
DVU2842	type II DNA modification methyltransferase	-2.66	-2.15	-2.32	-1.82	Y
DVU3359	hypothetical protein	-2.72	-2.40	-2.61	-2.13	

* phage and transposon related and co-localized genes

Table 4. Gene expression profile of selected genes involved in amino acid biosynthesis and metabolism

Gene ID	Gene description	Gene expression change, Log ₂ ratio			
		30 min	60 min	120 min	240 min
DVU0085	tryptophan synthase, beta subunit	1.36	1.75	1.90	2.45
DVU0086	hypothetical protein	1.18	1.88	0.84	3.00
DVU0285	imidazole glycerol phosphate synthase	0.88	1.03	1.10	0.15
DVU0286	imidazoleglycerol phosphate synthase, cyclase subunit	1.79	1.39	1.53	0.66
DVU0339	D-isomer specific 2-hydroxyacid dehydrogenase family	1.01	0.85	1.20	1.24
DVU0460	predicted phospho-2-dehydro-3-deoxyheptonate aldolase	-0.08	0.04	0.15	0.57
DVU0461	predicted 3-dehydroquinase synthase	0.36	0.11	0.54	0.42
DVU0462	chorismate mutase/prephenate dehydratase	-0.04	0.26	0.73	0.47
DVU0463	3-phosphoshikimate 1-carboxyvinyltransferase	0.52	0.34	0.93	0.46
DVU0464	prephenate dehydrogenase	-0.04	0.12	0.56	1.02
DVU0465	anthranilate synthase, component I	0.87	0.20	0.51	0.48
DVU0466	anthranilate synthase, glutamine amidotransferase component	0.18	0.37	0.08	0.07
DVU0467	anthranilate phosphoribosyltransferase	1.16	0.70	1.18	0.97
DVU0468	indole-3-glycerol phosphate synthase	1.01	0.85	1.41	1.32
DVU0469	N-(5-phosphoribosyl)anthranilate isomerase	0.72	0.93	1.27	0.99
DVU0470	tryptophan synthase, beta subunit	0.61	0.66	0.71	0.74
DVU0471	tryptophan synthase, alpha subunit	0.99	1.16	1.15	1.31
DVU0663	cysteine synthase A	0.83	1.31	1.65	0.82
DVU0890	homoserine dehydrogenase	1.33	1.55	1.69	1.19
DVU1466	acetylglutamate kinase	1.43	1.01	1.36	1.04
DVU1585	vitamin B12-dependent methionine synthase family protein	1.53	1.66	1.27	1.00
DVU1609	dihydrodipicolinate reductase	1.31	1.27	1.13	0.73
DVU1610	glutamine-dependent NAD ⁺ synthetase	1.51	1.09	1.52	1.37
DVU2981	2-isopropylmalate synthase	0.49	0.65	0.48	0.34
DVU2982	3-isopropylmalate dehydratase, large subunit, putative	0.89	0.87	1.11	0.70
DVU2983	3-isopropylmalate dehydratase, small subunit	0.60	0.76	1.03	0.30
DVU2984	conserved hypothetical protein	0.96	0.97	1.30	1.00
DVU3048	aspartate-semialdehyde dehydrogenase	1.09	1.30	1.73	1.50
DVU3371	5-methyl-homocysteine S-methyltransferase	0.54	1.55	2.18	1.87

Figure 1.

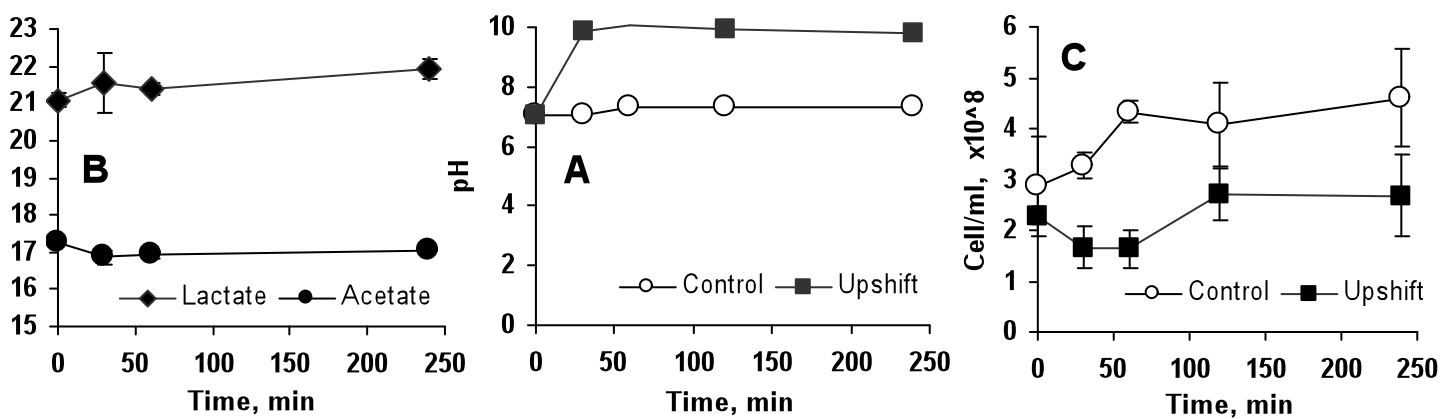


Figure 2.

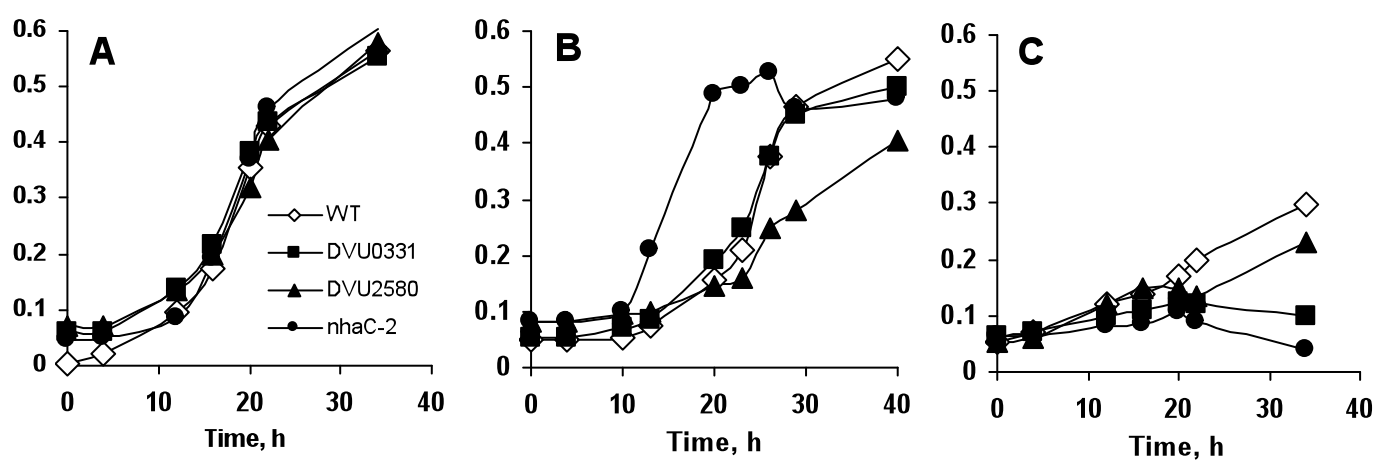


Figure 3.

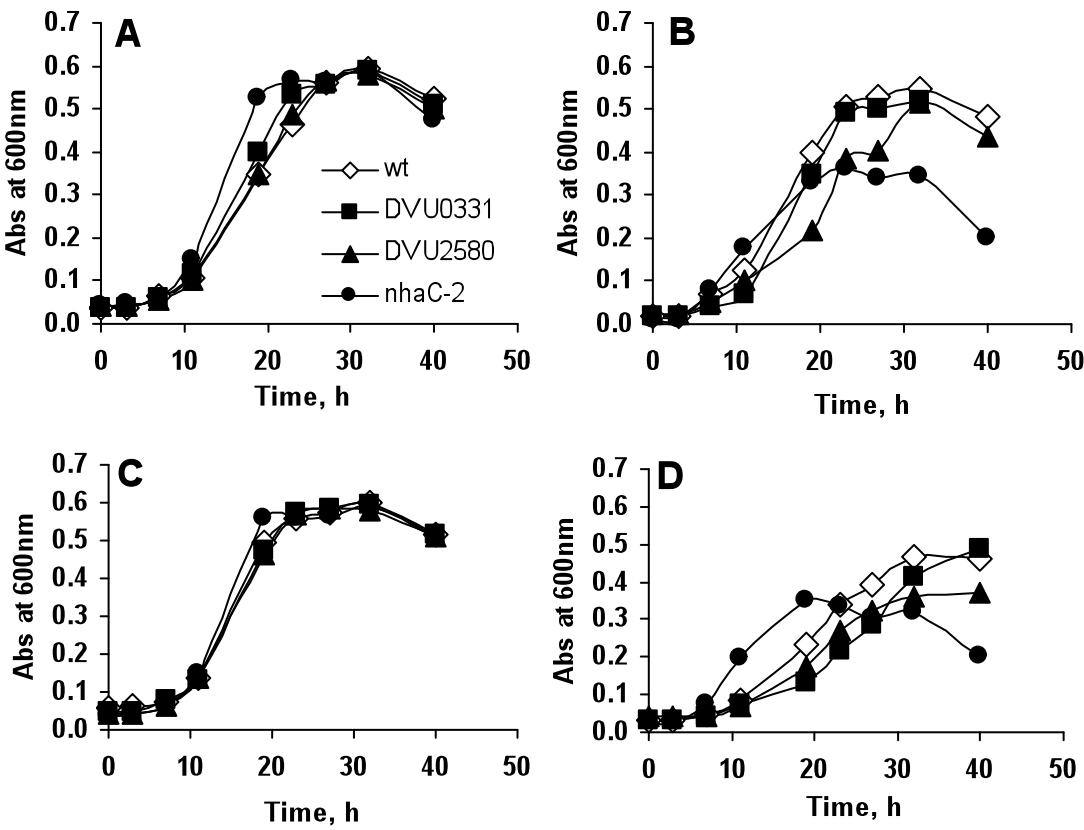


Figure 4.

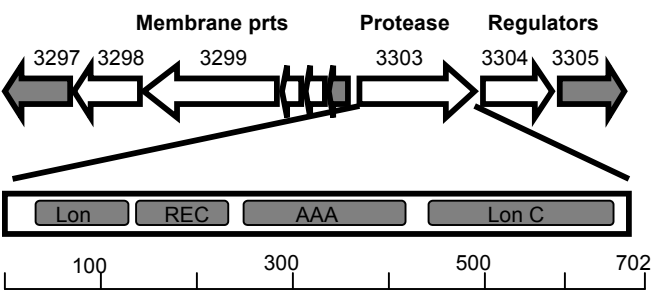
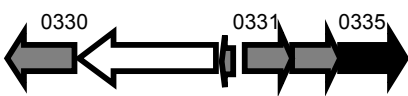
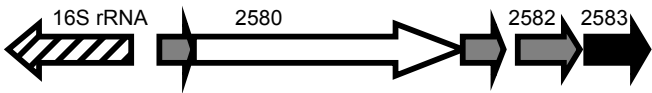


Figure 5.



Gene ID	Protein assigned function	Cellular process
DVU0330	Response regulator	Signal transduction
DVU0331	Histidine kinase	Signal transduction
DVU0332	Hypothetical protein	Unknown
DVU0333	Hypothetical protein	Unknown
DVU0334	D-alanine-D-alanine ligase	Cell envelope
DVU0335	3-deoxy-D-manni-octulosonic-acid transferase	Cell envelope

Figure 6.



Gene ID	Protein assigned function	Cellular process
DVU2579	Containing TPR repeats	Signal transduction
DVU2580	Histidine kinase	Signal transduction
DVU2581	Response regulator	Signal transduction
DVU2582	Response regulator, TetR family	Signal transduction
DVU2583	Lipoprotein	Cell envelope

Figure 7.

